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# ON THE MECHANISM OF PEROXIDASE-CATALYZED CHEMILUMINESCENCE FROM ISOBUTYRALDEHYDE

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SUMMARY. The reaction of isobutyraldehyde with dissolved oxygen catalyzed by horseradish peroxidase has been studied from the standpoint of determining the rate-limiting factor under a variety of conditions. Chemiluminescence from the product triplet acetone and rate of oxygen uptake were determined in simultaneous experiments. The reaction is initiated by the peracid obtained from the uncatalyzed autoxidation of the aldehyde; and under certain conditions the amount of peracid is also rate-limiting in the steady-state portion of the reaction. Under other conditions the total amount of enzyme and under still others the rate of formation of enol from the parent aldehyde controls the rate.

Although much progress has been made (1,2) the mechanisms of peroxidase-catalyzed aerobic oxidations are not fully understood. Chemiluminescent reactions such as obtained in the IBAL[1]/$O_2$/HRP system offer a unique opportunity to contribute to an understanding. The latter reaction has been used to probe transfer of electronic energy in biological systems and to induce photobiological processes in the absence of light (3,4). In previous studies of the IBAL/$O_2$/HRP system the emphasis has been on optimizing conditions for production of triplet acetone and oxygen uptake (3,5). Here we probe for rate-limiting factors under a wide variety of experimental conditions.

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[1]Abbreviations: HRP, horseradish peroxidase; IBAL, isobutyraldehyde.

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## MATERIALS AND METHODS

The analytical grade reagents were purchased from the following sources: HRP (type VI) from Sigma; $K_2HPO_4$, $KH_2PO_4$ and $K_4P_2O_7$ from Merck; $H_2O_2$ from Carlo Erba. IBAL (Janssen Chimica, 98%) was dried over $CaCl_2$ and distilled carefully using a Vigreux column flushed with $N_2$ (b.p. 64°C). IBAL was used in ethanol (1:5 and 2:5). [HRP] was determined using $\varepsilon = 1.02 \times 10^5$ $M^{-1} cm^{-1}$ at 403 nm (6) and $[H_2O_2]$ using the peroxidase assay (7).

$O_2$ consumption was followed with a Yellow Springs Instrument Model 53 Oxygen Monitor and a sample holder constructed in the laboratory. Chemiluminescence was measured in counts per 5 s in a Hamamatsu TV Photon Counter C-767. The typical reaction mixture unless otherwise stated was: 0.10 M phosphate buffer, 40 mM pyrophosphate buffer both at pH 7.4, 42 mM IBAL, 1.6 μM HRP and 0.46 M ethanol. Deionized water was used. The total volume of the reaction mixture was 2.6 mL. All experiments were conducted at (35.0 ± 0.1)°C. The reaction was initiated by addition of aliquot amounts of IBAL solution followed by HRP to the remainder of the pre-thermostated reaction mixture.

## RESULTS AND DISCUSSION

The HRP dependence of the rate of light emission and rate of $O_2$ uptake in the steady state phase is shown in Fig. 1. The two curves appear superimposable within experimental error. However the inset shows clearly that at low [HRP] the two curves are linear and parallel. The light emission curve passes through the origin whereas the $O_2$ uptake curve has a finite intercept with the ordinate. Dependence of light emission and $O_2$ uptake

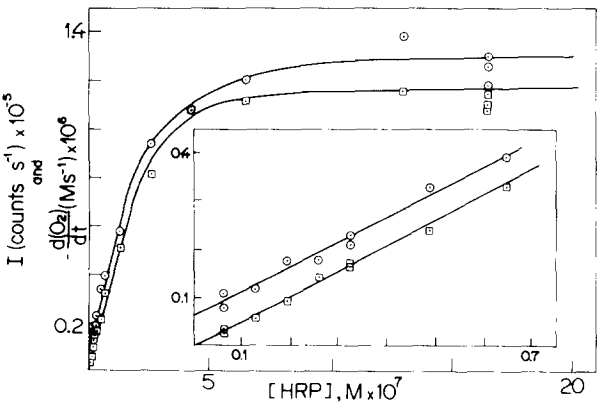


Fig. 1 - Effect of [HRP] upon the rate of light emission, I, and $O_2$ uptake. [IBAL] is 67.7 mM. For other conditions see Materials and Methods. ⊡ Rate of light emission (proportional to triplet acetone concentration); ⊙ rate of $O_2$ uptake. The inset shows the values at low [HRP] on expanded scales.

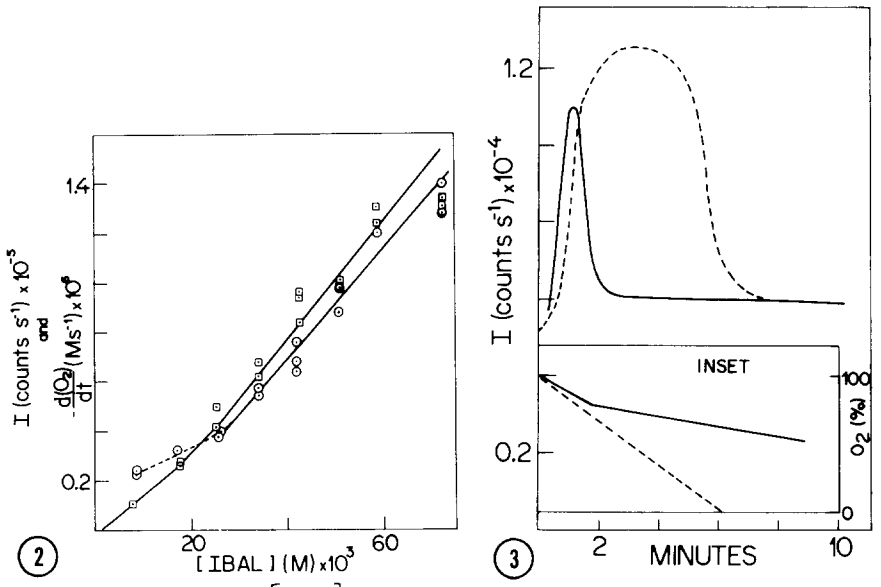


Fig. 2 - Effect of [IBAL] upon the rate of light emission, I, and $O_2$ uptake. ⊡ Rate of light emission; ⊙ initial rate of $O_2$ uptake.

Fig. 3 - Effect of $[H_2O_2]$ upon the rate of light emission, I (left ordinate) and in the inset $O_2$ uptake (right ordinate). The time scale is the same for the inset. [IBAL]= 8.5 mM. (——) no added $H_2O_2$; (----) $[H_2O_2]$= 1 x $10^{-5}$ M.

upon [IBAL] in the steady state phase in shown in Fig. 2. The two curves are plotted on the same ordinate scale as in Fig. 1. There is a definite lag in the rate of both reactions at small [IBAL]. Finally, in Fig. 3, the effect of added $H_2O_2$ upon the time course of both light emission and $O_2$ uptake is shown for low [IBAL].

Depending upon the experimental conditions three different factors appear to control the extent of the reaction. For lower values [HRP] is rate-limiting (Fig. 1). The chemiluminescent reaction is known to occur by catalyzed oxidation of the enol form of IBAL (3,5). The first-order formation of enol (8) would appear to be rate-limiting at larger [HRP] (Fig.1). Accordingly when $H_2O_2$ was added to the reaction mixture under the conditions of larger [HRP] as in Fig. 1 no rate enhancement was observed. Enol formation also appears rate-limiting for

larger [IBAL] (Fig. 2). However the lag phase in Fig. 2 is not first order in [IBAL]. For small [IBAL] a striking effect is observed from added $H_2O_2$ (Fig. 3).

The reaction of dissolved $O_2$ with IBAL catalyzed by HRP appears to occur spontaneously. Typically there is an initial burst in luminescence followed by a steady-state phase in which $O_2$ is consumed in an approximately zero order process (3). Luminescence occurs from triplet acetone with high efficiency (3,5). There are three distinct processes which must be considered in the overall reaction. (i) The uncatalyzed autoxidation of the keto form of the aldehyde. Essential features have been determined (see for example (9)). A chain reaction occurs in which many molecules of peracid are produced for each initiation event. The peracids are excellent oxidizing substrates for HRP comparable to $H_2O_2$ (10,11) which explains the "spontaneous" nature of the HRP-catalyzed reaction. Despite the precautions taken the IBAL still contained some initiating peracid. However for low [IBAL] the amount of peracid becomes rate-limiting (Figs. 2 and 3). (ii) The HRP-catalyzed autoxidation of the keto form of the aldehyde. In this process HRP would replace the photochemical or thermal reaction which generates the initiating R-$\dot{C}$=O radical. A conventional peroxidatic cycle would be followed by the chain reaction which occurs in the uncatalyzed autoxidation of the keto form. However since many molecules of peracid are formed for each initiation of the peroxidase cycle by one peracid molecule, the catalyzed autoxidation would be a branching chain reaction. The potential for an explosive process appears to be in competition with the luminescent reaction. Evidently the keto form of the aldehyde is so much less reactive than the enol that it is not able to compete. Confirmatory evidence is obtained in blank experiments. These show that within rather large experimental

error the difference between oxygen uptake and light emission (inset of Fig. 1) can be accounted for entirely by the uncatalyzed autoxidation of the keto form of the aldehyde. (iii) The peroxidase-catalyzed autoxidation of the enol form of the aldehyde. This reaction leads to triplet acetone formation and luminescence. It dominates the overall process as shown by estimates of total light emission (3) and product analysis (5). The luminescent reaction is initiated by the peracid. This is followed by hydrogen atom abstraction from the enol and addition of $O_2$ to form the α-peroxy radical of the aldehyde (3). Then hydrogen atom abstraction occurs within the enzyme (5,12) leading to an intermediate which decomposes to triplet acetone and formic acid. A detailed kinetic analysis is forthcoming.

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REFERENCES

1. Yokota, K. and Yamazaki, I. (1977) Biochemistry 16, 1913-1920.
2. Smith, A.M., Morrison, W.L. and Milham, P.J. (1982) Biochemistry 21, 4414-4419.
3. Bechara, E.J.H., Faria Oliveira, O.M.M., Durán, N., Casadei de Baptista, R. and Cilento, G. (1979) Photochem.Photobiol. 30, 101-110.
4. Cilento, G. (1980) Acc.Chem.Res. 13, 225-230.
5. Faria Oliveira, O.M.M., Haun, M., Durán, N., O'Brien, P.J., O'Brien, C.R., Bechara, E.J.H. and Cilento, G. (1978) J. Biol.Chem. 253, 4707-4712.
6. Ohlsson, P.-I. and Paul, K.-G. (1976) Acta Chem.Scand. B30, 373-375.
7. Cotton, M.L. and Dunford, H.B. (1973) Can.J.Chem. 51, 582-585.
8. Chiang, Y., Kresge, A.J. and Walsh, P.A. (1982) J.Am.Chem. Soc. 104, 6122-6123.
9. Zaikov, G.E., Howard, J.A. and Ingold, K.U. (1969) Can.J. Chem. 47, 3017-3029.
10. Dunford, H.B. (1982) Adv.Inorg.Biochem. 4, 41-68.
11. Dunford, H.B. and Stillman, J.S. (1976) Coord.Chem.Rev. 19, 187-251.
12. Rivas-Suárez, E. and Cilento, G. (1981) Biochemistry 20, 7329-7333.